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Single cell metabolomics

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Recent discoveries suggest that cells of a clonal population often display multiple metabolic phenotypes at the same time. Motivated by the success of mass spectrometry (MS) in the investigation of population-level metabolomics, the analytical community has initiated efforts towards MS-based single cell metabolomics to investigate metabolic phenomena that are buried under the population average. Here, we review the current approaches and illustrate their advantages and disadvantages. Because of significant advances in the field, different technologies are now at the verge of generating data that are useful for exploring and investigating metabolic heterogeneity.

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Introduction

Quantitative metabolomics, the technology for large-scale quantification of intracellular metabolite concentrations, is a powerful tool in systems biology research that has recently led to a series of interesting findings (e.g. [1–4]). Because of the metabolome's chemical diversity, mass spectrometry (MS) is the analytical method of choice [5]. In addition to analytical challenges, quantitative metabolomics as required for addressing (systems) biology questions poses significant challenges in sample processing. One important challenge is the need to preserve the original metabolome during sample processing, which is often difficult because of the presence of enzymes in the sample and the fast metabolic turnover rates.

For sensitivity reasons, current metabolomics methods require samples that contain a large number of cells. However, cell populations are not necessarily homogeneous. Besides genetic differences, several other sources for population heterogeneity exist, of which several are also known to cause metabolic differences. Today, methods capable of resolving differences in metabolite levels on the single cell level are provided, within limits, by molecular sensors such as FRET sensors [6,7] or aptamer-based technology [8•,9]. Both types of molecular sensors, however, are difficult to develop, are limited to specific analytes, and quantitative analyses (e.g. in terms of mol/L) are hardly possible with them. Laser-induced fluorescence, as introduced by Dovichi for single cell proteomics, is limited to fluorescent compounds or labelled species [10,11]. In addition, all these existing methods share the limitation that they can never be extended to the ''-omics'' level, that is to measuring a large number of metabolites at the same time. They will thus not be applicable to discovery type research and research that requires a large number of metabolites to be measured in the same cell.

Because of the success of mass spectrometry in population-level metabolome analyses, the analytical community has recently made great strides towards single cell level metabolite analyses (for a review, see [12]). So far, however, hardly any new biological insight has been generated from these endeavours. In this *Current Opinion* paper, we will thus not only review the current status of MS-based single cell metabolomics but also discuss which of the different approaches will have the best chance to be useful for addressing (systems) biological questions. We intend to update the interested biology community on how far single cell metabolomics has been developed by analytical chemists, and help the analytical community to guide their efforts towards the needs of the future users in (systems) biology.

Why single cell metabolomics?

The metabolome is arguably the most sensitive measure of a cellular phenotype [13]. Thus, metabolomics is not only an essential experimental tool for metabolism-related research: we anticipate that it will also become a powerful tool for general screening studies, because of its potential to uncover phenotypic differences in a very sensitive manner. The power of metabolomics can be exploited in population-level measurements, but as cell populations are not necessarily homogeneous, it will be even more informative to measure at the single cell level.

There are multiple reasons for cellular heterogeneity: cells can be genetically different, can experience a different microenvironment, might have had a different history, are in different developmental or cell-cycle

stages, or of different age. Because techniques probing metabolism at the single cell level are still largely lacking, the research community has almost no knowledge about the metabolic phenotypes of individual cells in heterogeneous populations. However, from a limited number of experiments with sorted cell populations and from some other reasoning, it is clear that significant differences in the metabolome are to be expected. For example, from population-level metabolomic measurements on cell-cycle-synchronized yeast cells, it was found that significant changes in gene regulation and metabolite levels occur during the cell cycle [14]. Also, the age of a yeast cell was argued to influence the metabolic phenotype in clonal cells: In cells that were sorted according to their age, dramatic metabolic differences were found [15–17]. Synchronizing as well as sorting cells likely affects the metabolome, due to fast metabolic turnover rates. Typical sorting methods do not account for any metabolic changes that occur during the process, such that the available measurements might not reflect the original metabolome. Thus, even such studies, where information on the metabolic phenotypes of certain subpopulations already exists, would also tremendously benefit from single cell metabolomics technology.

In addition to the abovementioned sources for heterogeneity, stochasticity-induced phenotypic heterogeneity was identified a few years ago as an additional source of cell-to-cell variability [18,19]. Because of low copy numbers of specific biomolecules, certain processes at the gene and protein expression level are inherently stochastic and can cause random fluctuations in the abundance of biomolecules. These fluctuations can be exploited by a number of regulatory feedback mechanisms to create multiple distinct and coexisting phenotypes even from isogenic cells in the same environment and in cells with the same history (cf. reviews cited above). The two prerequisites for stochasticity-induced phenotypic heterogeneity [20,21] are met in practically every biomolecular network. Thus it is very likely that the currently known cases of such heterogeneity merely represent the tip of the iceberg of all of these cases [22].

In fact, this type of heterogeneity was also found to occur in metabolic systems: the well-known lactose utilization system in *Escherichia coli* was found to display an ''all or none'' type of behaviour, where single cells stochastically transit between the two states [23]. Another example is the galacatose ultilization network in yeast displaying bimodal patterns in the expression of the GAL family genes responsible for galactose metabolism [24]. A recent report about the presence of several global feedback loops overarching metabolic and transcriptional regulation in *E. coli* [25] indicates that in fact many more metabolically different stable phenotypes could be uncovered — if we were only able to experimentally probe metabolism of individual cells. Metabolically different phenotypes may underlie the medically highly relevant microbial phenotypes of persister cells [26], dormant cells [27] or small colony variants [28].

Overall, a technology to measure metabolite levels in single cells would be an excellent tool for firstly discovery of metabolic differences in individual cells (for which likely semi-quantitative methods will be sufficient) and secondly system biology endeavours that aim at generating an understanding about the emergence of such phenotypic or genetic heterogeneity (for which likely more quantitative methods will be required).

Challenges for single cell metabolomics

Single cell metabolomics poses enormous challenges: First, it has to deal with minute quantities of analytes. In *E. coli*, a bacterium with about 1 fL of volume [29], even highly abundant glycolytic intermediates, which are present in the low mM concentration range, require detection sensitivity in the low attomole range. In the budding yeast, with a volume of about 65 fL [29], amounts in the two- to three-digit attomole range must be detected. In mammalian cells, with a 500 fL volume [29], metabolites would be expected in the two-digit femtomole range (assuming similar metabolite concentration levels as in microbes). Compared to the metabolite amounts that are typically used for classical population-level metabolomics (nanomoles), single cell metabolomics for *E. coli* has to handle amounts that are approximately 10^9 times lower. Unlike in single cell genomics and proteomics, amplification of analyte and/or highly sensitive fluorescence measurements on labelled compounds cannot be used for single cell metabolomics.

Recently, the limits of detection for metabolites using mass spectrometry have been lowered from typically femtomoles to the low attomole range which is the range that is required for single cell metabolomics [30••]. However, even if such small quantities can be detected, quantification is still problematic, and so is handling of the minute sample quantities (originating from an individual cell). Transfer of a cell (content) to the mass spectrometer, ideally without any losses, harbours significant challenges, especially because the sample processing needs to conserve the original metabolome (which can, for example, be distorted if enzymatic activity is still present in the sample). Furthermore, quantitative mass-spectrometric analyses typically require the metabolites to be separated from cell debris, proteins, and salts, to reduce ion suppression.

In order for a single cell metabolomics technology to be useful for (systems) biology research, it is important that the technology can either measure a wide range of metabolites such that the technology could be used for phenotypic screening on the single cell level or can measure and quantify fewer metabolites in a targeted

manner. Further, the technology should reproducibly provide at least semi-quantitative data. For this, it is important that not only the actual analysis step is quantitative, but also the sample processing is done in an appropriate way. Finally, because single cell metabolomics technology is expected to reveal differences between few individual cells, the technology also needs to generate sufficient measurement throughput to allow meaningful statistical analyses of the data will.

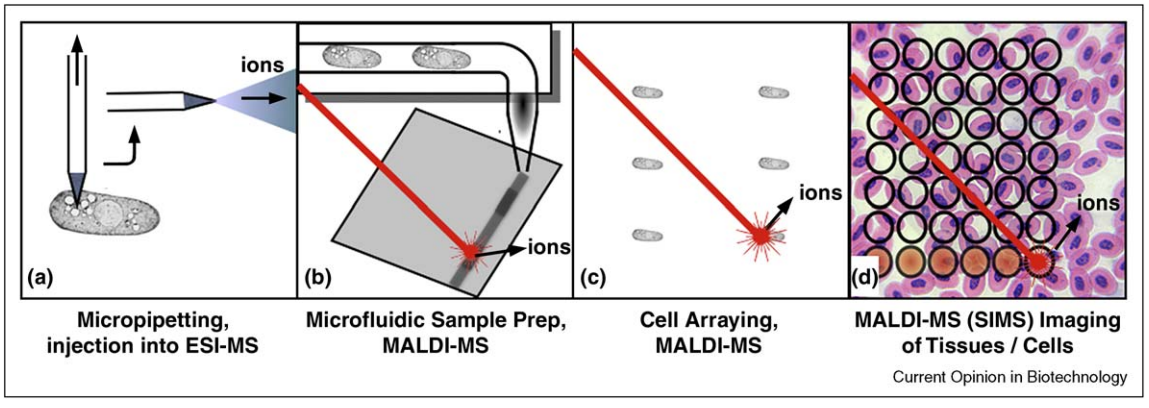
MS-based approaches for single cell metabolomics

On the population level, mass spectrometry has become the key enabling technologies for metabolomics, providing high-resolution data. In recent years, an increasing number of papers have been published on the topic of MS-based single cell metabolomics. Almost exclusively, papers on this topic have appeared in analytical journals and have focused on the technology development; real biological insight has not yet been generated. In the following, we will illustrate the different approaches pursued (Figure 1) and will discuss to what extent these technologies have the potential to generate data relevant for (systems) biology.

(a) *Sampling the cell contents with a micropipette, followed by injection into a mass spectrometer using a nano-electrospray ionization (nano-ESI) source.* This approach, which is probably only suitable for very large cells, has been pursued by Masujima [31] who also coined the term ''live single cell video-mass spectrometry'' for it, to indicate that the cytoplasm, or in some cases even subcellular material, is sampled under a video microscope with a small pipette that can then be directly used for generating ions via nano-ESI. Although the same group has presented some (unpublished) work on robotic handling of the pipetting process, this approach is hardly amenable to high-throughput operation: only a few cells can be measured per hour. Unless the throughput can be dramatically improved, this approach will thus not really be relevant for systems biology, because it will be likely impossible to generate statically sound data on cellular heterogeneity. On the other hand, this is the method that has so far yielded by far the greatest variety of signals, with more than a dozen signals that were clearly distinct from the abundant background signals. For example, metabolites such as histamine, serotonin, and leukotriene B4 could be detected in individual mast cells. Identification by tandem mass spectrometry was done off-line, on samples stemming from a large number of cells. In addition to metabolites, enzymes are also sucked into the needle, such that distortion of the original metabolome can be an issue.

(b) *Sample preparation on a microfluidic chip, followed by deposition on a sample plate for (matrix-assisted) laser desorption/ionization (MALDI or LDI) mass spectrometry.* For this approach, single cell organisms are first processed on a microfluidic chip with steps for quenching, lysis, and separation of metabolites from the rest of the cell content. The cell content is then transferred to a mass spectrometer with a suitable interface. Conceptually, this approach represents a downscaling of the classical metabolomics approach. The complete setup has not yet been realized, but many different units required for the microfluidic chip have been developed separately, such as lysis (see, for example, [32]), impedance-based cell size measurement [33], CE separation (e.g. [34]). Also, it has already been demonstrated for metabolites including UDP, ADP, GDP, UTP, ATP, GTP, acetyl-CoA, and butyryl/isobutyryl-CoA that the sensitivity for MALDI-based MS detection of metabolites will be sufficient to reach the single yeast cell level [30••] and a suitable technology for sample deposition (''writing'' onto a MALDI plate) was also proposed [12,35,36] allowing for convenient off-line mass-spectrometric analysis. Coupling of the

Figure 1



Schematics of the four MS-based approaches for single cell metabolomics.

microfluidic device to an ESI MS [37•,38] is also possible, which, however, requires on-line MS analysis. Once the overall operation of this technology has been demonstrated, it is likely that this technology has the potential to generate high-throughput data in an automated way.

(c) *Sample arraying*. In this approach, suitable for single cell organisms, a sample plate for LDI or MALDI is covered by a solvent-repelling (''omniphobic'') coating that has been patterned to form a dense checkerboard arrangement of hydrophilic recipient sites approximately 50–200 μm in size, matched to the size of the laser focus. These spots are ''anchors'' for small volumes of liquid that will automatically form a checkerboard pattern of quite monodisperse sample droplets after spreading a cell suspension onto this patterned plate. If an appropriate concentration of cells is used, spreading of the sample will result in the deposition of $\leq$1 cell (in rare cases, two cells) per recipient site. An advantage of this approach is that the size of the cells to be analyzed may be distinctly smaller than the recipient site. The plate can be cooled to stop the metabolism instantaneously upon applying the cell suspension. Application of a MALDI matrix in an organic solvent will then lyse the cells and extract the compounds of interest for analysis by MALDI. So far, high-abundance metabolites (e.g. ADP, GDP, UTP, ATP, GTP and GDP-Glc) have been detected by negative ion mode MALDI-MS in small algae and in single yeast cells. We have hints that this sample presentation mode also facilitates quantitation, because the signals from recipient sites containing two cells are about twice as high as from those with only one cell. This approach provides true high-throughput operation: the sample deposition is an automated, parallel process, and the readout of the spectra is fast, limited only by the speed of the MS instrument ($\geq$2 spots per second, that is analysis of 1000s of cells/hour) [39••].

(d) *Imaging mass spectrometry*. Many modern mass spectrometers have imaging capabilities, with a spatial resolution of typically ≈50 μm (MALDI or LDI), and ≈1 μm (secondary ion mass spectrometry, SIMS), as well as relatively fast acquisition speed [40•,41]. Unless the cells are much smaller than 50 μm, MALDI-MS imaging thus has the capability to yield single cell analyses of compounds including metabolites. Imaging MS is most often applied to analyzing tissues. Interesting work in this area has been published by various groups. Sweedler and coworkers have pioneered the quantitative analysis of neurotransmitters and neuropeptides in single neurons of rats and the sea snail *Aplysia californica* [42]; the latter, however, are really gigantic. Interesting sample preparation protocols have been developed by the same group [43•]. Another recent example is MS imaging of the distribution of secondary flavonoid metabolites such as kaempferol, quercetin, isorhamnetin, and their glycosides in individual plant tissue cells [44]. A spatial resolution of 10 μm was reached with state-of-the-art LDI instrumentation. SIMS imaging of cells is capable of even higher spatial resolution, but has not really been extended to detecting metabolites. With this technology the distribution of ions such as Na^+, K^+, Ca^{2+}, as well as cationized cholesterol, lipids, or their fragments, that is molecules present at cell surfaces has usually been imaged [41]. It remains to be shown that SIMS imaging will also be able to detect intracellular metabolites. SIMS is notorious for suffering from ionization matrix effects, and such it seems unlikely that it will yield quantitative data.

Conclusion

The importance of metabolism for health and disease is currently being rediscovered, and combined with the recent discovery of stochasticity-induced phenotypic bistability as one source of population heterogeneity, it is clear that technologies for metabolomics on the single cell level will be required.

The analytical field has started to develop technologies for MS-based single cell metabolomics, as seen from the increasing number of publications in this area. However, so far, all efforts in single cell metabolomics seem to remain only within the analytical community. The challenge for the near future will be to bring together the efforts of the analytical community with current interests in the area of (systems) biology, such that the technological efforts can be guided towards the needs of future users in biology. Here, the analytical community will likely face requests for proper sample handling procedures (i.e. procedures that conserve the original metabolome) and for targeted measurement of certain specific metabolites. In current single cell metabolomics papers often rather exotic classes of metabolites were reported — presumably simply because these were the only metabolites that were easily detected. For application of single-cell metabolomics technology to biological research, however, it likely will be necessary to specify metabolites (or metabolite classes) to be measured.

We feel that ultimately the approach to couple a microfluidic unit to a mass spectrometer has the highest potential to deliver relevant data for systems biology. Here, the major challenges will be — apart from solving the quantification issue — to integrate all the single steps into a whole system. We hope that within the next two or three years such systems will become available.

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